FINAL REPORT

I. **CONTRACT NUMBER:** N000014-80-C-0310

II. **TITLE:** BIOCHEMICAL CONTROL OF MARINE FOULING

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IV. **PERIOD:** March 1, 1980 - January 14, 1988

V. **OBJECTIVES:**

The objectives of research supported by this contract have been: (1) to identify and characterize the biochemical signals, receptors and transducers, and their mechanisms of action, that control the settlement, metamorphosis, and recruitment of larvae of marine invertebrates onto surfaces, and thus control marine fouling; and (2) based on these results, to identify new strategies for countermeasures that are specific, non-toxic, and environmentally safe.

VI. **INTRODUCTION:**

Although marine fouling is initiated, in several cases, by the accretion of a thin molecular and microbial layer (microfouling), it is the attachment of larger organisms (macrofouling) that is responsible for the principal increase in drag on vessel hulls, increase in fuel consumption, stress and deterioration of piers, pilings and cassions, and the reduced efficiency of heat-exchangers in the marine environment. Virtually all marine organisms that contribute to macrofouling are recruited to immersed surfaces as larvae (or propagules) settling from the plankton.

With the large diversity of macrofouling organisms encountered, and the heterogeneity of environments and conditions under which fouling occurs, an understanding of the basic biochemical mechanisms controlling larval recruitment and fouling is needed for the development of more effective and widely applicable countermeasures. [The nature of this problem is broadly stated in the recent report of the NSF, entitled "Emergence of a Unified Ocean Science," which concludes: "The world ocean is heterogeneous and variable on many different scales, and most of the basic underlying scientific generalities are yet to be formulated."] In the case of macrofouling, and the control of larval recruitment, we recently have identified what we believe to be one of the basic underlying generalities. The identification of this principal underlying mechanism may offer the possibility for development of a new generation of antifouling countermeasures that are specific, widely effective and non-toxic. Other potentially useful spin-offs also are described.

VII. SYNOPSIS OF RESULTS:

We have identified, purified and characterized a new class of signal molecules, previously undescribed, that are required for induction of settlement, attachment, metamorphosis and subsequent growth of *Haliotis* larvae (and larvae of a number of other organisms) on immersed surfaces in the marine environment. These signal molecules, initially associated with specific algal proteins, recently have been purified as active small fragments of M ~1,000 daltons. The active moiety apparently is a conjugate of the amino acid neurotransmitter, GABA (γ-aminobutyric acid) or a close structural homolog. GABA and its homologs also induce the fouling sequence, in a stereochemically specific manner. We have identified a similar protein-conjugated amino acid-derived neurotransmitter-mimetic inducing signal, containing derivatives of the transmitter DOPA (dihydroxyphenyl-alanine), that is required for induction of the fouling sequence and cementation of the gregarious, cementing, tube-building worm, *Phragmatopoma* (a major macrofouling organism). Thus, in larvae of species representing 2 different phyla, a basic similarity has been found between the signal molecules required for induction of the fouling sequence. These inducing signal molecules usually are found only in association with recruiting surfaces in the marine environment; detection by the recruited larvae is mediated by contact-dependent chemosensory recognition.

We recently have identified a large group of marine and freshwater bacteria that produce the signal molecule required for induction of the fouling sequence in *Haliotis* and related species. As this group of bacteria has been shown to be tractable for DNA recombination, cloning, and plasmid-directed mutagenesis, use of these methods is expected to facilitate our further analysis of the structure and mechanism of action of the microbial fouling-signal molecule, and the control of its synthesis and recognition.

Chemosensory receptors responsible for recognition of GABA-related algal and microbial signals controlling the fouling sequence in *Haliotis* have been identified, and shown to be biochemically and functionally similar to GABA receptors in post-synaptic membranes. These larval receptors appear to be located externally on specialized epithelial chemosensory nerve endings; the receptors are glycoprotein; stereochemically highly specific; present in approximately 10^{11} copies/larva; and show a high chemical affinity (K_D ~10^{-7} M) for the required signal. The activity of these receptors is regulated allosterically by ligand-induced changes. These receptors also are regulated by: facilitation by water-borne diamino acids (lysine, etc.); habituation; endogenous factors; and development.

Transducers essential for responsiveness to the GABA, algal and microbial signals have been found to include: induced movements of specific ions across the chemosensory membrane; induced synthesis of cyclic AMP; induced secretion of a glycoprotein; and induced enzyme changes. Of these, the most important for development of practical countermeasures appears to be the induced transmembrane movement of specific ions, which traverse ion-channels through the chemosensory membrane controlled by the GABA receptors. Studies with ions, ion-channel blocking agents and ionophores reveal that recognition and binding of GABA-related signal molecules by these receptors opens chloride channels, thus resulting in an efflux of chloride ions across the membrane to produce an excitatory depolarization. This excitatory depolarization of the membrane transduces the fouling signal from a chemical signal detected in the external environment to a neuronal impulse. This mechanism of transduction of the chemical signals required for induction of fouling appears to be essential and widely applicable to many species. We have shown that species for which the required chemical signals are not yet known can be induced to settle, attach and metamorphose simply by causing excitatory ionic depolarization, and that blockade of this essential step may offer a practical means for general control.

Two different classes of chemosensory receptors mediating the induction of settlement and metamorphosis (ISM) of *Haliotis* (gastropod) larvae have been specifically labeled, identified, and characterized directly at the biochemical level. We have succeeded in the purification and *in vitro* characterization of the larval receptors, signal transducers and

amplifiers controlling ISM in response to environmental chemical signals. Two distinct pathways, mediated by separate groups of sensory receptors and signal transducers, have been found to control the ISM of *Haliotis* larvae in response to two different classes of environmental chemical signals (GABA-mimetic peptides associated with specific algal surfaces, and a specific group of amino acids dissolved in seawater). The Morphogenetic (Trigger) Pathway, activated by binding of the GABA-mimetic peptide to its cognate receptors on the larval epithelium, produces a signal that is transduced by changes in cyclic AMP, calcium, and ion flux across the chemosensory membrane; the resulting excitatory depolarization of this membrane produces the neuronal signal that triggers ISM. The second pathway, called the Regulatory (or Amplifier) Pathway, is activated by specific amino acids in the water-borne dissolved organic material (DOM). These facilitating amino acids act through a separate group of epithelial receptors and membrane-associated transducers (including a G protein, diacylglycerol, and a protein kinase) to covalently modify a component that increases the output of the Morphogenetic Pathway. The result of this interaction between the Amplifier and Trigger Pathways greatly increases the sensitivity of the larvae, priming them for enhanced settlement and metamorphosis in response to low concentrations of the surface-associated inducer.

Larvae of the gregarious tube-building polychaete, *Phragmatopoma californica*, have been demonstrated to respond to DOPA-containing peptide inducers of settlement and metamorphosis present in the tube-cement of the conspecific adults. Specific fatty acids found in the conspecific tubes also induce settlement and metamorphosis. Recent results demonstrate: (a) the involvement of a morphogenetic signal transduction pathway (involving adenyl cyclase, cyclic AMP, and excitatory membrane depolarization) similar to that in *Haliotis* larvae; and (b) that morphogenetic DOPA analogs of the tube-cement are active in the natural environment, inducing substratum-specific settlement and metamorphosis in the ocean. This represents one of the few instances in which a defined chemical signal shown to induce larval settlement and metamorphosis in the laboratory also has been shown to cause ISM in the ocean. These results demonstrate the persistence of a defined, surface-associated chemical cue in the ocean, and the use of chemical recognition by the larvae (in this environment) in the selection of sites for settlement and metamorphosis.

Based on these results, we have explored the development of a new generation of fouling countermeasures that are specific and non-toxic. Blockers of the receptor we have found to satisfy these criteria include non-inducing structural analogs of the inducing signal molecules, and specific lectins that block the signal-binding site. The high stereochemical specificity of each receptor suggests, however, that the principal value of such agents will be for analytical purposes. Blockers of the transducers appear to be far more widely useful; those we have characterized in our laboratory include ionic (electrochemical) mechanisms, and agents active at membrane ion-channels.

In Section VIII and IX, below, details are presented of results with larvae of the mollusc *Haliotis*, which we developed as an experimental model system, and with larvae of *Phragmatopoma*, a significant macrofouler.

VIII. RESULTS WITH *HALIOTIS*, A MODEL SYSTEM:

1. Development of a Uniquely Tractable Experimental System:

Larvae of the gastropod mollusc, *Haliotis rufescens* (red abalone) have proved uniquely tractable for experimental analyses of the molecular and cellular mechanisms of signal recognition, signal transduction, and subsequent responses controlling larval settlement and metamorphosis in response to signal peptides produced by specific algae and phylogenetically related bacteria (Morse et al., 1979, 1980a,b,c, 1984; Morse and Morse, 1984a). The larvae (0.2 mm diam.) are lecithotrophic, exhibit an absolute requirement for the morphogenetic inducer, and, with procedures we developed for the control of spawning and uniform larval

development (Morse et al., 1977a; 1979), can be produced conveniently in large numbers (suitable for receptor purification and analysis) on a regular basis throughout the year.

In our work completed over the past few years, we have modified and improved our procedures to enable us to produce 10-20-fold more larvae, on a weekly basis, than previously possible. As a result, we now typically produce 3-9 x 10^6 sibling larvae competent for metamorphosis each week. These larvae thus can be produced in quantities sufficient for molecular and biochemical analyses (e.g. Trapido-Rosenthal and Morse,1986), with high reproducibility of their developmental timing and morphogenetic specificity (Trapido-Rosenthal and Morse, 1985, 1986, 1987; Baxter and Morse, 1987; Yool et al., 1986). To facilitate quantitative analyses of morphogenetic induction, we recently have developed a sensitive and specific biochemical procedure for measurement of the calcium-binding conchiolin (shell) peptide, whose synthesis is activated (in the differentiating and proliferating cells of the mantle) quickly after larval recognition of the inductive stimulus (Cariolou and Morse, 1987). Results obtained with this experimentally tractable non-mammalian model system have proven widely applicable to a large number of other species of several different phyla (Jensen and Morse, 1984; Yool et al., 1986; Morse, 1985, 1987a; and see below).

2. Further Characterization of the GABA-mimetic Peptide Inducers of Settlement and Metamorphosis:

We previously had shown that a family of oligopeptides (M ~ 600-1300 daltons) produced by CCA, other red algae and cyanobacteria induces settlement and metamorphosis in *Haliotis* larvae and the larvae of certain other marine invertebrates (Morse et al, 1984; Morse and Morse, 1984a,b; present project: Morse, 1985, 1986, 1987b). We recently have found that oligopeptides exert their morphogenetic effect by interaction with a specific group of epithelial GABA (-aminobutyric acid) receptors on the larvae (see below), and that these peptides also bind strongly to $GABA_A$ receptors purified from mammalian brain (Morse et al., in preparation).

The signal peptides are found initially associated with the phycobiliproteins that are uniquely present as photosynthetic accessory pigments in the cyanobacteria and red algae. We have used high-resolution gel-filtration chromatography, followed by ion-exchange and HPLC to separate the morphogenesis-inducing peptides from the phycobiliproteins, and to purify these peptides approximately 20,000-fold, from several species of cyanobacteria and red algae. The resulting oligopeptides all have been found to be structurally closely related, with each containing the same unusual (as yet unidentified) basic amino acid residue (Morse, 1985, 1987b, and Morse et al., manuscript in preparation). Both the intact peptides and this unusual amino acid residue (but no other residues derived from the peptide) have been found to mimic the action of the amino acid neurotransmitter, GABA, both in the binding to larval receptors and subsequent induction of larval settlement and metamorphosis (Trapido-Rosenthal and Morse, 1986; Morse 1985, 1986, 1987b; Morse et al., in preparation), and in the high affinity binding to GABA receptors purified from mammalian brain (Morse, 1985, 1987b; Morse et al., in preparation). The signal molecule responsible for induction of larval settlement and metamorphosis thus is a GABA-mimetic oligopeptide produced by cyanobacteria and red algae (Morse, 1985, 1987b). We recently have succeeded in producing a panel of monoclonal antibodies against the GABA-mimetic oligopeptides.

3. First Successful Labeling and Direct Characterization of Chemosensory Receptors Controlling Larval Settlement and Metamorphosis:

One of the major accomplishments of our project has been the specific radiochemical labeling of the chemoreceptors controlling settlement and metamorphosis in *Haliotis*. This labeling has made possible the direct biochemical characterization of these receptors and their down-regulation. It also has facilitated the analysis of another mechanism of regulation, and has made the start of receptor purification possible, as described below.

Recognition of the morphogenetic signal molecules by *Haliotis* larvae is mediated by specialized chemosensory receptors that control the subsequent behavioral and developmental metamorphosis of the planktonic larvae (Morse et al., 1980b,c; Morse, 1984a, 1985). Preliminary evidence, including sensitivity to competitive blockade by specific lectins, suggested that these receptors may be glycoproteins located on the externally accessible larval epithelium (Morse, 1984a; Baloun and Morse, 1984). The *Haliotis* receptors are stereochemically specific for GABA, certain GABA analogs, and the GABA-mimetic oligopeptides produced by the cyanobacteria and red algae (Morse et al., 1980c, 1984; Trapido-Rosenthal and Morse, 1986). In both their order of affinities for various GABA analogs, and their sensitivity to inhibitors, these morphogenesis-controlling GABA receptors differ from other previously described GABA receptors (Trapido-Rosenthal and Morse, 1986, 1987). We recently have succeeded in directly labeling these receptors, and further characterizing their binding properties and their regulation (Trapido-Rosenthal and Morse, 1986). These are the first such chemoreceptors controlling the settlement and metamorphosis of any marine invertebrate to be successfully labeled and characterized directly.

The receptors are specifically labeled with tritiated (-)-baclofen(-chlorophenyl-GABA). This labeling is saturable, reversible (with no change in the radioactive baclofen molecule), and stereochemically specific. Scatchard and log-logit analyses of binding data indicate that there are approximately 10^{10} receptors per larva; the affinity of these receptors for (-)-baclofen is reflected by a $K_D = 3 \times 10^{-7}$. [Our original results indicated a $K_D = 10^{-6}$ M (Trapido-Rosenthal and Morse, 1986); the lower value we now obtain, indicating a slightly higher affinity, is a reflection of recent improvements in our experimental procedures, making possible the cleaner separation of binding to the morphogenesis-controlling receptors from the contribution to apparent binding from lower-affinity transport systems (Markell and Morse, in preparation).] Identification of these labeled receptors with those controlling metamorphosis is based on four independent criteria (Trapido-Rosenthal and Morse, 1986): (1) The specificity and affinity of the receptors determined from direct labeling and competition studies closely parallels those identified by measurements of the induction of metamorphosis, with a correlation coefficient of 0.97; the order of effectiveness of small-molecule GABA analogs in both binding and morphogenetic induction is: (-)-baclofen > GABA > muscimol > 3-aminopropanesulfonic acid. These results indicate that the affinity of a given ligand for this receptor determines its effectiveness as an inducer of metamorphosis. (2) Radioactive baclofen specifically bound to the receptors is shed from the larvae after 20 hours, at the time corresponding to the metamorphic abscission and shedding of the apical tuft sensory cilia and other specialized larval structures that are jettisoned in response to the metamorphic induction. [We recently have obtained independent and direct evidence that the receptors are contained on some of these cilia (Baxter and Morse, unpublished observations; see below)] (3) Both the availability of the receptors for labeling, and the ability of the larvae to respond (by settlement and metamorphosis) to the GABA-mimetic peptide, GABA, or GABA analogs can be down-regulated in parallel (see below). This down-regulation or desensitization of the larvae is caused by premature exposure to GABA (prior to the development of competence to undergo metamorphosis). Our initial labeling studies showed that down-regulation resulted from a 70% reduction in the number of receptors, with no alteration in the affinity or Hill coefficient of those receptors remaining. (Trapido-Rosenthal and Morse, 1985, 1986, 1987). Recent improvements in the specificity of labeling of the morphogenesis-controlling receptors (discussed above) now show that down-regulation removes or inactivates 100% of the morphogenesis-controlling receptors, leaving other receptors and transport sites unaffected (Markell and Morse, in preparation). (4) The principal morphogenetic signal molecule purified from marine cyanobacteria and red algae is a GABA-mimetic oligopeptide (see above); this purified peptide competes with baclofen for the labeled receptor, confirming our earlier suggestion that the morphogenetic peptide acts at a population of specialized GABA receptors on the larvae. This oligopeptide is the most effective ligand yet found for both direct interaction with the labeled receptor, and the induction of metamorphosis; in both assays, it is effective with a $K_D = 10^{-7}$ M. (The foregoing data are summarized from Trapido-Rosenthal and Morse, 1986; except as otherwise noted).

4. Down-Regulation of the Receptors, and its Potential Significance:

We discovered that premature exposure of the developing *Haliotis* larvae to GABA or GABA analogs for periods as short as 3 h, prior to the development of larval competence for metamorphosis, followed by removal of the exogenous GABA, is sufficient to desensitize larvae to subsequent morphogenetic induction by GABA and its analogs at a time when sibling larvae normally become responsive to induction by GABA (Trapido-Rosenthal and Morse, 1985, 1986, 1987). This down-regulation was first shown to result from a 70% reduction in the number of detectable chemosensory receptors, with no change found in the affinity or the Hill coefficient of the remaining receptors (see above) (Trapido-Rosenthal and Morse, 1986). Recent improvements in the specificity of our receptor labeling reveal that 100% of the morphogenesis-controlling receptors are removed or inactivated by down-regulation, with no effect on other receptors or transporters (Markell and Morse, in preparation).

As further evidence that down-regulation occurs specifically at the level of the receptor, we have demonstrated that the GABA-insensitive larvae still show normal induction of settlement and metamorphosis in response to both an increase in external potassium ion concentration (producing an excitatory depolarization), and an increase in internal cyclic AMP concentration (Trapido-Rosenthal and Morse, 1986, 1987). Increases in internal cyclic AMP and membrane depolarization both have been identified as early post-receptor events in the morphogenetic signal transduction pathway (see below); either alone is sufficient to induce metamorphosis, bypassing the requirement for receptor activation. That the habituated larvae still respond normally to these signals thus confirms that down-regulation is confined to an inactivation of the receptors; the post-receptor pathway of signal transduction and end-organ response is completely unaffected.

Down-regulated larvae progressively recover responsiveness to GABA and GABA analogs, in parallel with their recovery of detectable receptors (Trapido-Rosenthal and Morse, 1986, 1987; Markell and Morse, in preparation). This mechanism thus may have adaptive significance, in that it may enhance the dispersion of larvae in the plankton before settlement (Trapido-Rosenthal and Morse, 1986, 1987), as first suggested by Hadfield (1977). The more general significance of these results is that they demonstrate that the sensitivity of marine invertebrate larvae to morphogenetic signal molecules can be down-regulated by reduction in the number of chemosensory receptors available for interaction with the molecules that induce settlement and metamorphosis. In this respect, chemosensory receptors for environmental and morphogenetic signals are for the first time demonstrated biochemically to respond to habituation in a manner similar to neuronal and hormonal receptors (Trapido-Rosenthal and Morse, 1986).

5. Signal Transduction by Cyclic AMP, Calcium, Protein Phosphorylation and Membrane Depolarization:

We previously had shown that activation of the larval chemosensory receptors by GABA or GABA-mimetics produces a signal transduced by an increase in intracellular cyclic AMP, changes in intracellular calcium, and an efflux of chloride ions resulting in excitatory depolarization of the chemosensory membrane (Morse et al., 1980a; Baloun and Morse, 1984). We now have shown that increases in cyclic AMP produced by addition of the cAMP-phosphodiesterase inhibitors isobutylmethylxanthine (IBMX) or theophylline, by activation of adenyl cyclase with forskolin, or by direct addition of dibutyryl-cyclic AMP are sufficient to induce metamorphosis, bypassing the requirement for receptor activation (Trapido-Rosenthal and Morse, 1986; Baxter and Morse, 1987). We also recently have found that metamorphosis can be induced with the calcium ionophore, A23187, and that induction by GABA is sensitive to verapamil, a calcium channel blocker, lending support to the involvement of calcium in the signal transduction pathway (Baxter and Morse, in preparation). We recently have observed that a specific protein in the larvae (possibly the sensory membrane ion channel protein) is phosphorylated soon after activation of the larval receptor (Baxter and Morse, in preparation).

Our previous evidence indicated that binding of the GABA-mimetic signal molecule to the larval receptor leads to the opening of chloride channels in the membrane, causing an efflux of chloride that results in an excitatory depolarization or firing of the chemosensory cell (Baloun and Morse, 1984). We have demonstrated that this simple mechanism is widely applicable to the control of metamorphosis in larvae of several other species and phyla (Yool et al., 1986).

6. Recent Finding that G Protein, Diacylglycerol and Protein Phosphorylation Regulate Settlement and Metamorphosis in Response to a Second Group of Signal Molecules in the Water-Column: Potential Adaptive Significance:

We recently have found that the settlement and metamorphosis of *Haliotis* larvae in response to low concentrations of the GABA-mimetic peptide can be facilitated, or permanently amplified, by as much as 1,000-fold, by brief exposure of the larvae to any of several diamino acids structurally related to lysine, that are found in the water-column DOM (dissolved organic material) (Trapido-Rosenthal and Morse, 1985, 1987). Direct labeling and characterization of the larval receptors (as described in section 3, above) reveals that this amplification of sensitivity to inducers of metamorphosis occurs without alteration in the number or affinity of the larval receptors for the morphogenetic inducers (Trapido-Rosenthal and Morse, 1986). Recent evidence shows that this amplification of the morphogenetic response occurs at a post-receptor level, independent of inducer binding at the GABA and GABA-mimetic peptide chemoreceptor (Trapido-Rosenthal and Morse, 1987; Baxter and Morse, 1987).

We have found that transduction of the amplifying signal is mediated by a regulatory pathway that is separate from the morphogenetic pathway (Baxter and Morse, 1987; Morse, 1987a). Activation of this regulatory pathway produces a permanent "up-regulation" or sensitization to settlement-inducing stimuli, which recent work indicates is the result of a covalent protein phosphorylation that itself is not morphogenetically inductive. The protein phosphorylation activated by this regulatory pathway is distinguishable from the protein phosphorylation induced by activation of the morphogenetic pathway (Baxter and Morse, in preparation). Our evidence suggests that this regulatory pathway involves a diacylglycerol second messenger, and that the level of this second messenger is regulated by a guanine nucleotide binding protein (G protein) that is controlled by a separate class of chemoreceptors. Activation of the regulatory pathway does not by itself induce settlement or metamorphosis of the *Haliotis* larvae, nor does blockade of the regulatory pathway inhibit normal responsiveness of the larvae to inducers of metamorphosis (Baxter and Morse, 1987).

These results thus establish the existence of separate regulatory and inductive pathways controlling larval metamorphosis in response to two classes of exogenous chemical signals from the environment. The regulatory pathway, operating independently through a G protein-diacylglycerol cascade apparently controlled by facilitating diamino acids in the water column, can amplify the larval responsiveness to inducers of metamorphosis. This mechanism may have adaptive significance in the recognition and selection of favorable habitats for settlement and metamorphosis of the larvae. Whereas the fine-scale spatial specificity of settlement and metamorphosis in association with specific algae or bacteria can be determined by the availability and larval recognition of GABA-mimetic peptides produced by these potential associates, the responsiveness of the larvae to these inducers can be controlled by an independent regulatory pathway. Activation of this G protein-diacylglycerol pathway by certain dissolved amino acids in the water column, which may serve as indicators of the quality of the larger-scale environment (e.g., the availability of nutrients, etc.) thus can prime the larvae, enhancing their responsiveness to (bacterial and algal) inducers of settlement in potentially favorable areas (Morse, 1985; Trapido-Rosenthal and Morse, 1985, 1987; Baxter and Morse, 1987). We have suggested that similar pathways, based on exogenous control of a G protein-diacylglycerol cascade, may govern responsiveness to stimuli in other sensory and developmental systems (Baxter and Morse, 1987). The larger significance of

these findings (and those described in the preceding sections) lies in the fact that they provide the most complete picture yet available of the molecular mechanisms controlling the recognition of, and responsiveness to, environmental chemical signals and their regulation of settlement and metamorphosis in marine invertebrate larvae. These results establish that the mechanisms of signal recognition, signal transduction, receptor regulation, and facilitation that control larval metamorphosis in response to exogenous chemical signals are closely related to the mechanisms involved in recognition of, and responses to, neurotransmitters and hormones.

IX. RESULTS WITH *PHRAGMATOPOMA*, A SIGNIFICANT MACROFOULER:

1. Cementing Polychaetes are Major Macrofoulers:

Cementing polychaetes of the family Sabellariidae, barnacles of the genus *Balanus*, and mussels (genus *Mytilus*) represent major groups of hard and firmly attached macrofoulers that produce major and costly problems for Naval operations worldwide. The recruitment of these hard macrofoulers causes significant increases in vessel drag and fuel consumption, deterioration of hulls and structures immersed in the ocean, and reduction in the efficiency of heat exchangers. Identification of the environmental and surface factors that control the initial events leading to the recruitment of these macrofoulers would make possible the predictive modeling of macrofouling, and the more effective development of practical (and possibly non-toxic) countermeasures.

2. DOPA-mimetic Inducers from Marine Adhesives and Bacteria:

The finding that certain naturally occurring inducers of larval settlement and metamorphosis are functionally and structurally related to peptide conjugates of amino acid neurotransmitters extends beyond the case discussed above. We recently have found that gregarious settlement and metamorphosis of larvae of the tube-building polychaete, *Phragmatopoma californica*, are induced with high specificity by the fresh cement of the tubes made by conspecifics; this material consists of DOPA-containing quinone-tanned proteins, (Jensen and Morse, 1988). DOPA (dihydroxyphenylalanine, an amino acid neurotransmitter) and its analogs also induce settlement and metamorphosis of *P. californica* larvae (Jensen and Morse, 1984).

Phragmatopoma californica is a fouling organism uniquely suitable for further analysis of the control of settlement, adhesion and metamorphosis by DOPA-mimetic inducers. This species forms massive fouling aggregations of many thousands of individuals, housed in tubes of cemented sand. Gregarious recruitment of larvae from the plankton is dependent upon larval contact-dependent chemosensory recognition of active inducer that is uniquely associated with the fresh cement (i.e., of the most distal portion) of the tubes of the conspecifics (Jensen and Morse, 1984). Similar findings, with the larvae of related Sabellariids, had been made earlier by Wilson (1968). The requirement of the larvae for contact with the fresh tube-cement of their conspecifics (apparently reflecting the time-dependent oxidative inactivation of the inducer; Jensen and Morse, unpublished observations) ensures the fail-safe induction of settlement and metamorphosis only in response to recognition of a biochemical signal that is a reliable indicator of habitats that have supported recent success (growth) of the adult conspecifics. By providing adult *Phragmatopoma* in the laboratory with clean glass beads with which to extend their tubes, we have been able to isolate the inducing substance - the adherent fresh cement - in nearly pure form (Jensen and Morse, 1984, 1988).

Beads with this cement efficiently induce larval settlement and metamorphosis of *P. californica*, while control glass beads (with or without primary organic films or marine bacteria) all are inactive (Jensen and Morse, 1984). As in the case of the *Haliotis* inducer, this settlement-inducing molecule is firmly associated with the recruiting substrate, and is not readily released in a soluble, freely diffusible form (Jensen and Morse, 1984). Hydrolysis of the

partially purified inducer (obtained on the glass beads), and analysis by HPLC, reveals that the settlement-inducing cement is a polyphenolic protein, cross-linked by quinone-tanning of its DOPA residues (Jensen and Morse, 1988). DOPA and various DOPA analogs and oligopeptides of defined sequence also prove active in triggering settlement and metamorphosis of *P. californica*. The inducer thus appears fundamentally similar to that of *Haliotis* settlement and metamorphosis, being a peptide- or protein-conjugate of an amino acid neurotransmitter (DOPA) (or of a closely related structural analog of DOPA) (Morse, 1983, 1984a, 1985a,b). The ability to conveniently obtain the natural DOPA-mimetic inducer of settlement and metamorphosis of this fouling organism in relatively pure form and sufficient amounts, as described above, has made possible its complete purification and structural analysis (Jensen, Morse and Waite, in preparation).

3. The Adhesive Cement of *Phragmatopoma*:

As explained above, the fresh adhesive cement of the gregarious fouling worm, *Phragmatopoma californica*, has been obtained in our laboratory in nearly pure form, attached to glass beads (Jensen and Morse, 1984). This material is of special interest, because it serves not only as a quick-setting and strong adhesive to surfaces and structures in the ocean, but also serves as a potent inducer of gregarious larval settlement and metamorphosis, thus responsible for the explosive growth of attached concretions of macrofouling animals.

Our recent analysis of this material reveals the cement to be a DOPA-rich quinone cross-linked protein, closely analogous to the byssus adhesive of the mussel that had been characterized by Waite and his colleagues (Jensen and Morse, 1988; Jensen, Morse and Waite, in preparation). This conclusion is based on our studies of the composition of acid hydrolysates of the *Phragmatopoma* cement in which amino acids were identified and quantified by HPLC, and DOPA and related catecholamines identified and quantified by HPLC and sensitive electrochemical detection (Jensen and Morse, 1984, 1988). The very high content of DOPA that we observed in the cement was verified by two additional independent and specific methods of analysis: (1) specific colorimetric determination with acid-molybdate; and (2) conventional amino acid and catecholamine analyses by ion-exchange chromatography, and determination with ninhydrin, performed in collaboration with Dr. Herbert Waite, at the University of Connecticut and University of Maryland.

Results of these analyses indicate that the cross-linked cement contains ca. 18 residues of DOPA per 1000 amino acid residues; this very high DOPA content is similar to that reported by Waite for the byssus of *Mytilus*. The *Phragmatopoma* cement also contains approximately 26% each of serine and glycine, hydrophilic residues that compete effectively with water and form extensive β-pleated sheet conformations similar to those in silk. Indeed, the high serine and glycine contents are very similar to those in sericin, a natural cement secreted with silk fibers (Jensen and Morse, 1988). Such DOPA-rich, extended-sheet scleroproteins are uniquely effective adhesives in the marine environment for several reasons (Waite, ms. in preparation) including: (a) provision by DOPA residues of sites for multiple and rapidly formed cross-links (both covalent and non-covalent), that are enzymatically initiated, and then autocatalytically strengthened in proportion to turbulence and oxygenation, thus producing rapidly setting cements with high tensile and adhesive strength; (b) hydrophilic competition for surfaces with water; (c) formation of exceptionally strong DOPA chelates with metals, bonding virtually irreversibly and nonspecifically to metals in stone, cement, and metallic surfaces ($K_A > 10^{23}$); (d) maximization of surface contact as a result of extended (non-helical) sheet-like conformation; and (e) resistance to microbial degradation, seawater, and abrasion.

In further studies carried out in our laboratory in collaboration with Dr. Waite, we determined that the mode of secretion and crosslinking of the *Phragmatopoma* cement is similar to that previously characterized for mussel byssus (Jensen, Morse, and Waite, in preparation), and suggested by ONR researchers to occur in the cementation of barnacles. The *Phragmatopoma* system, however, offers distinct advantages for the experimental analysis of

this process. Our recent studies demonstrate that DOPA-rich precursors of the cement are stored in both mandibular and caudal glands of *Phragmatopoma*, and that secretion of a specific DOPA-oxygenase together with these precursors initiated the process of cross-linking and strong adhesion (Morse, Jensen, and Waite, unpublished observations). The experimental tractibility of the *Phragmatopoma* system (cf. Jensen and Morse, 1984, 1988), and the ability to utilize artificial beads of varying and precisely defined compositions, will facilitate both physical and biochemical analyses of the initial and rate-determining processes governing the adhesion of these cements (which serve bifunctionally as adhesives and inducers of gregarious recruitment.

X. NEW INHIBITORS OF LARVAL SETTLEMENT, ADHESION AND METAMORPHOSIS:

Using the larval *H. rufescens* system as an experimentally tractable model, we have begun investigations of the specificities, effectiveness, and mechanisms of action of various classes of inhibitors of larval recruitment, adhesion and metamorphosis which are in current use or may have future value as antifouling control measures. While the induction of larval settlement and metamorphosis of *H. rufescens* proves quite sensitive to heavy metals, the lack of specificity of these widely toxic agents is readily apparent (Morse, et al., 1979b). Thus, although GABA-induced settlement, attachment, and metamorphosis can be effectively blocked by various copper compounds, these substances also prove toxic and lethal to the swimming (i.e., non-induced) larvae at concentrations only slightly greater than those required to block induction of settlement (Morse et al., 1979b). This lack of specificity is reflected in the general environmental toxicity of the copper compounds, which deleteriously affect many species beyond the target fouling community.

In similar assays, a number of halogenated hydrocarbons proved somewhat more specific. These compounds inhibit the induced settlement, attachment, and metamorphosis of *H. rufescens* larvae at relatively low concentrations, while producing little or no acute toxic effect or interference with the swimming of uninduced larvae (Morse et al., 1979a,b). The apparent selectivity of the halogenated hydrocarbons suggests that these substances may block normal induction of settlement, attachment and metamorphosis by inhibition of membrane-associated receptor sites. Nevertheless, the long-term environmental, physiological and genetic effects of these compounds, and their accumulation and persistence in marine food-chains, make their use for the control of marine fouling impractical.

We therefore have attempted to design and test new, potentially specific (nonhazardous, environmentally safe) biochemical inhibitors of the recruitment and fouling reaction-sequences. In this effort, we have concentrated first on the design and testing of agents which specifically block the larval receptors and transducers essential for normal induction of the fouling process (see above).

We have found that certain noninducing stereochemical analogs of GABA can block induction of settlement, attachment, and metamorphosis of *H. rufescens* larvae (Morse et al., 1980c; Morse, 1984a). The fact that this inhibition is competitive with GABA, and that the stereochemical requirements for inhibition indicate specificity for a molecular structure closely analagous to GABA (in chain-length, terminal substituents, etc.), suggest that these noninducing analogs act by direct inhibition of specific chemoreceptor sites to which the required inducer otherwise would bind. Although the compounds which are effective in receptor-blockade are relatively non-toxic, we find a high species-specificity of such inhibition as well, suggesting that such compounds would have little general usefulness in the control of marine fouling. Nevertheless, these compounds have been useful in our analyses of the larval receptors themselves (Morse et al., 1980c). These findings also indicate that it may be possible to design species- (or group-) specific control measures using agents which block larval receptors required for the recognition of inducers of the fouling sequence.

A potentially more general method for receptor-blockade is suggested by our experiments with defined lectins. These glycoproteins, purified from biological sources, show specificity of binding to various other glycoproteins which are commonly constituents of membranes and membrane-associated receptor-sites; specificity of binding is determined by the structure of the glycosyl units projecting from the target membrane glycoproteins. Using various purified lectins of known specificity as probes of the structure of the chemoreceptors in *H. rufescens* larvae, we have found that it is possible to specifically and completely inhibit GABA-induced settlement, attachment, and metamorphosis of these larvae by exposure to low concentrations of any of three different lectins with identical binding specificity for glucosyl and mannosyl determinants (Morse 1984a). Five other lectins, each with specificities for other glycosyl determinants (N-acetylglucosaminyl, N-acetylgalactosaminyl, galactosyl, and fucosyl sugar residues) are inactive in this blockade. Evidence that the inhibition of recruitment responses by the three effective lectins is caused by binding to - and blockade of - required receptor sites is seen from the protection of these larval sites by prior exposure to inducer (GABA) or the active sugar determinants (glucose and mannose), as well as from the direct competition observed between the inhibitory lectin and the inducer (Morse 1984a). As discussed above, these findings suggest the epithelial (i.e., external) location of the receptor sites (accessible to the impermeant macromolecular lectins), and provide useful information on the structural determinants of the membrane-associated glycoprotein receptors. The experimental use of lectins thus may facilitate the design of practical synthetic blocking agents with structures and affinities specifically tailored for interaction with characterized larval receptor sites.

Inhibition of the essential transducers that mediate translation of the biochemical signals controlling larval settlement and metamorphosis has been most promising to date. As described above, binding of the inducing signal molecules at larval chemosensory receptors activates a flux of ion movement across the chemosensory membrane, causing a depolarization capable of transducing the chemical signal from the environment to information propagated by the larval nervous system (Baloun and Morse, 1984; Yool et al., 1985). As a result, we have found it possible to specifically inhibit larval attachment with: (a) agents active at membrane ion channels, and (b) weak opposing electrochemical (ionic) gradients (Baloun and Morse, 1984). These findings may provide one of the first basic explanations for the observed effectiveness of weak electrical fields in the inhibition of fouling. In view of the apparent wide applicability of the ionic transduction of inducing signals (Yool et al., 1985), agents capable of inhibiting these essential transducers may provide the possibility of broad-spectrum effectiveness, coupled with environmental safety (Morse, 1984a; 1985a,b).

XI. PUBLICATIONS RESULTING FROM THIS CONTRACT:

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XII. GRADUATE STUDENTS:

Baxter, G. (Ph.D. anticipated 6/89)
Cariolou, M. (Ph.D. 6/85)
Jensen, R. A. (Ph.D. 6/86)
Morse, A. N. (Ph.D. 6/85)
Trapido-Rosenthal, H. G. (Ph.D. 6/85)
Yool, (Baloun), A. J. (Ph.D. 6/85)

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XV. INVENTIONS:

None.